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Splinkerettes—improved vectorettes for greater efficiency in PCR walking

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Vectorettes enable PCR amplification of DNA sequences which lie between a single known primer and a nearby restriction site. They have been applied in the isolation of end fragments from YAC recombinants (1) and also in PCR walking (2). Vectorettes consist of a double-stranded linker sequence with a central region of mismatch and a cohesive end suitable for ligation to restriction enzyme-digested DNA (Fig. 1). The vectorette primer employed in the PCR is exactly the same sequence as the mismatched portion of the upper strand, and therefore cannot anneal to and initiate priming from the vectorette until its complementary sequence has been synthesised by polymerase extension from the specific target DNA primer.

The specificity of the vectorette PCR reaction is, however, not absolute. Illegitimate products result from non-specific annealing of either primer and from a reaction we term 'end-repair' priming. End-repair priming involves the free cohesive ends of unligated vectorettes and inserts which are based on restriction sites that produce 5' overhangs. These ends are filled in during the first cycle of the PCR reaction. After the subsequent denaturing step, these ends are able to anneal to each other with sufficient stability to initiate priming (3). Extension across a vectorette sequence at either end of the insert molecule will result in the production of a sequence complementary to the vectorette primer, allowing exponential PCR amplification without involvement of the specific target DNA primer. Vectorette dimers will also be produced by end-repair priming, but will not be amplified in the PCR because the two halves will form a stable hairpin structure which will be refractory to amplification.

The splinkerette we describe here is designed to decrease end-repair priming. Rather than a central DNA mismatch, the splinkerette incorporates a hairpin structure on the bottom strand (Fig. 1). The primer is of the same sequence as the top strand and therefore, as with the vectorette, is unable to act as a primer until the complement of this strand has been synthesised. In a PCR reaction the free 3' end of the bottom strand will flip back on itself to form a hairpin and begin elongation further along the bottom strand. The resulting double-stranded structure is stable and is functionally removed from further reaction. It is therefore not able to cause end-repair priming. Furthermore, in the splinkerette system, only the top strand is available to act as a non-specific primer whereas in the vectorette system both the top strand (which after end-repair is four bases longer than that of the splinkerette) and the bottom strand could cause mispriming in this way. Splinkerettes are not kinased, and as such there is no

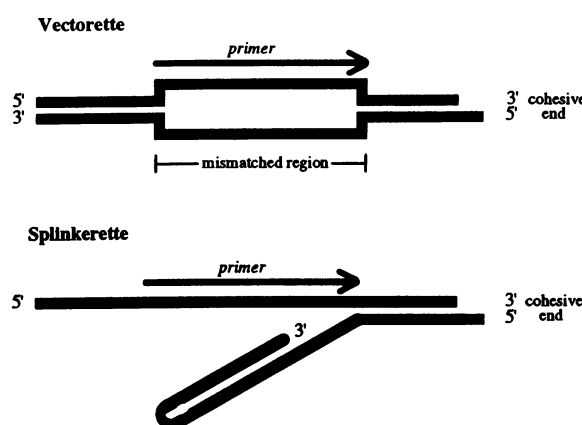


Figure 1. Schematic diagram of the vectorette and splinkerette.

covalent bond between the splinkerette bottom strand and the DNA to be amplified. This avoids elongation from the hairpin along the whole length of the insert molecule.

Here we demonstrate the practical advantages of splinkerettes over vectorettes by comparing the efficiency of both in producing unique products from the complex resource of total human genomic DNA. To enable direct comparison, splinkerettes and vectorettes were designed to be compatible with a single primer.

For small target PCR products (≤ 500 bp) it was found that the efficiency of both the splinkerette and vectorette was adequate to produce the expected products after one or two rounds of PCR (data not shown). However, Figure 2 shows the advantages of splinkerettes over vectorettes in the amplification of larger fragments where formation of the target product may be hindered by increased competition from end-repair primed artefacts. A 2.3 kb fragment from the WT1 gene (4) was successfully amplified only with the splinkerette. We ascribe the reproducible failure of vectorette PCR to amplify this fragment, and the smear of non-specific products instead resulting, to end-repair priming. The usefulness of splinkerette PCR has been further demonstrated by the routine isolation of long end fragments (up to 3.6 kb) from YAC recombinants (5).

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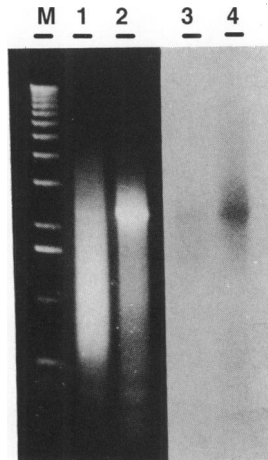


Figure 2. Comparison of splinkerettes and vectorettes in PCR walking. The target PCR product was a 2.3 kb fragment derived from the WT1 gene (3). Vectorettes were obtained from ICI. Splinkerettes were made by duplexing the top strand (5'-CGA ATC GTA ACC GTT CGT ACG AGA ATT CGT ACG AGA ATC GCT GTC CTC TCC AAC GAG CCA AGG-3') and the bottom strand (5'-GAT CCC TTG GCT CGT TTT TTT TTG CAA AAA-3') by mixing the oligonucleotides (150 µg/ml each) at 90°C in 10 mM Tris-HCl (pH 7.4), 5 mM MgCl₂ and leaving to cool to room temperature. Total human genomic DNA was digested with *Bam*HI and ligated with a 15 × molar excess of splinkerettes or vectorettes in 20 µl for 4½ h at room temperature. PCR combined Hot Start (6) and Touchdown (7) protocols and conditions were as follows: denaturation, 95°C for 30 s in the first cycle and 15 s thereafter; annealing, for 1 min at 71°C initially, decreasing by 2°C to 61°C per cycle and 61°C thereafter; extension, 72°C for 2 min (cycles 1–10), then 4 min (cycles 11–20) and finally 6 min (cycles 21–30). In the primary PCR reaction 1 µl of ligation product was amplified in 50 µl using 400 ng WT1 primer (5'-CCA AGG GCC GTG AGG ATA GCG GAA G-3') and 250 ng splinkerette/vectorette primer (5'-CGA ATC GTA ACC GTT CGT ACG AGA A-3'). Secondary PCR was performed using 1 µl primary PCR product, 400 ng internal WT1 primer (5'-GCA CGC AGG CAC TGG CCC CCG ACA T-3') and 250 ng internal splinkerette/vectorette primer (5'-TCG TAC GAG AAT CGC TGT CCT CTC C-3'). Tracks 1 and 2 show 10 µl vectorette and splinkerette secondary PCR product respectively resolved on a 1% agarose gel. The marker is the 1 kb ladder (Gibco-BRL). No product was visible after primary PCR for either the vectorette or the splinkerette. Tracks 3 and 4 show results of Southern blot analysis of tracks 1 and 2, respectively, with a 1.1 kb probe derived from within the target PCR product.

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